

Excitation Energy Transfer Study of the Spatial Relationship between the Carbonyl and Metal Cofactors in Pig Plasma Amine Oxidase¹

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9-Hydrazinoacridine irreversibly labeled pig plasma amine oxidase by covalent attachment to the active carbonyl cofactor. The visible absorption spectrum of the modified protein displays new absorption bands at 495 and 525 nm. Its emission spectrum exhibited maxima at 415 and 440 nm. In addition, both absorption and emission spectra were insensitive to pH changes between 6 and 10. Phase modulation fluorometry was used to determine fluorescence lifetimes of Zn²⁺- and Co²⁺-substituted acridinyl plasma amine oxidase. Energy transfer efficiency was 22%; the distance separating the Co²⁺ ion (in the copper binding site) and the acridine moiety (the amine substrate binding site) ranges between 11.7 and 14.7 Å. This work defines the proximity of the metal and substrate (and hence the carbonyl cofactor) and precludes any direct interaction between Cu²⁺ and pyrroloquinoline quinone or between Cu²⁺ and the substrate. © 1988

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Plasma amine oxidase⁴ (PAO)⁵ from pig belongs to a group of closely related soluble amine oxidases. This class is characterized as requiring cupric ions and an organic cofactor capable of reacting with carbonyl reagents (1-3). Pig PAO exists as a dimer with an apparent molecular

weight of 195,000, binds 2 mol of Cu²⁺ per dimer, and is completely inactivated by 1 mol of phenylhydrazine per mole of dimer (4).

The nature of the carbonyl containing prosthetic group has been an area of intense research. Many investigators have shown that 1 mol of phenylhydrazine or other carbonyl reagents stoichiometrically reacts with PAO and completely inactivates it, demonstrating that only 1 mol of functional cofactor exists per dimer (5-7). When incubated anaerobically, 1 mol of amine substrate binds to 1 mol of dimer (6, 8). Initially, pyridoxal phosphate or some modified pyridoxal had been proposed as the carbonyl cofactor (9). More recently, however, pyrroloquinoline quinone (PQQ) has been isolated from several amine oxidases and may be the active carbonyl containing cofactor (10-14).

In the catalytic mechanism, bound amine substrate is oxidized and then released as the corresponding aldehyde with the concomitant production of a reduced form of the enzyme. Subsequent binding of

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⁴ Amine:oxygen oxidoreductase (deaminating) (EC 1.4.3.6).

⁵ Abbreviations used: PAO, plasma amine oxidase; acr-PAO, 9-hydrazinoacridine-labeled PAO; PQQ, pyrroloquinoline quinone; dansyl, dimethylaminonaphthylene sulfonate; DCCH, 7-diethylaminocoumarin-3-carboxyhydrazide.

oxygen results in reoxidation of the enzyme and the release of ammonia and hydrogen peroxide (15). No evidence was found for the participation of copper-coordinated water in the hydrolysis of any intermediate, nor was copper the site of oxygen binding in pig plasma or pig kidney diamine oxidases (16, 17). However, kinetic and anion binding studies suggest that Cu^{2+} may play a role in the oxidation of a reduced form of the enzyme (18, 19), but the relationship between the organic prosthetic group and Cu^{2+} in the soluble amine oxidases remains unclear. The two cupric ions in pig PAO are distinguishable by EPR, suggesting that the dimer may not be symmetrical (16, 20, 21). When both copper ions are removed from bovine PAO, the enzyme is completely inactive (22, 23). When one copper is added back, the enzyme remains nearly inactive; the addition of a second copper restores the full activity (24). By contrast, when one copper is removed by diethyldithiocarbamate, the enzyme remains fully active (22).

Recently, ^{19}F NMR work has determined the spatial relationship between the amine binding site and one of the coppers in pig PAO. Using several trifluoromethylphenylhydrazine derivatives, the nearest Cu^{2+} was concluded to be in the same plane as the aromatic ring of the inhibitor and separated from it by a distance of approximately 12.5 Å (25). The present study was undertaken to confirm this distance using fluorescence energy transfer. 9-Hydrazinoacridine was used to inactivate the protein and to participate as the fluorescent energy donor. In this study, Zn^{2+} and Co^{2+} were used to replace Cu^{2+} in the metal binding site and permit the determination of energy transfer and distance calculations. Interactions between the cofactors and their roles in the catalytic mechanism may be inferred based upon their proximity.

EXPERIMENTAL PROCEDURES

Materials

Pig blood was obtained at a local slaughterhouse. Benzylamine and phenylhydrazine-HCl were purchased from Aldrich Chemical Co. and purified as

described by Falk (7). 9-Hydrazinoacridine was purchased from Kodak Chemical Co. Dansyl hydrazine was obtained from Sigma Chemical Co. 7-Diethylaminocoumarin-3-carbohydrazide (DCCH) and salicyl hydrazide were obtained from Molecular Probes. 1-Naphthalenemethylhydrazine and 2-naphthalenemethylhydrazine were prepared from their respective naphthalenemethylamines as described in (25). Chelex-100 resin was obtained from Bio-Rad Laboratories. Quinine sulfate was obtained from the National Bureau of Standards. Metal standards were purchased from Fisher Scientific Co. and nitric acid (double distilled from Vycor) was purchased from GFS Chemicals, Columbus, Ohio.

Methods

Enzyme purification. Pig plasma amine oxidase was prepared by the method described by Buffoni and Blaschko (1) as modified by Falk *et al.* (4). Activity was determined spectrophotometrically using benzylamine-HCl as substrate (4).

Reaction of pig PAO with carbonyl reagents. In general, pig plasma amine oxidase (2 mg/ml) was reacted at room temperature for periods up to 16 h with various amounts of carbonyl reagents (prepared as 10 mM stock solutions in absolute MeOH). Unbound reagents were removed either by several concentration-dilution cycles using Centricon-30 filter units (Amicon) or by dialysis. Phenylhydrazine was used to measure the extent of labeling with 9-hydrazinoacridine. The absorbance of 9-hydrazinoacridine-PAO (acr-PAO) was measured at 430 nm before and after the addition of excess phenylhydrazine. The net increase in absorbance at 430 nm was compared to the increase observed for a sample containing an equal concentration of unmodified PAO.

Preparation of metal-substituted pig PAO. All buffers used during metal removal and metal substitution procedures were prepared from analytical grade reagents and deionized water and made metal free by chromatography over a Chelex-100 column. Plasticware was used whenever possible and rinsed exhaustively with deionized water before use. Glassware was soaked in 25% nitric acid and rinsed with deionized water. Dialysis tubing was soaked in 10 mM 8-hydroxyquinoline-5-sulfonic acid and rinsed with deionized water. Metal content was assessed with a Perkin-Elmer Model 5000 atomic absorption spectrophotometer equipped with a Model 500 graphite furnace and a Model AS-40 autosampler. All samples had 2.0 g atoms of Cu^{2+} /195,000 g PAO before metal depletion and <0.1 g atom of Cu^{2+} afterward. Co^{2+} and Zn^{2+} -substituted PAO were prepared from metal-depleted PAO as described for the preparation of metal-substituted forms of bovine PAO by Suzuki *et al.* (23). The fact that the replacement metal ions return to the original copper-binding sites is evi-



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denced by the work of Suzuki (23). Using NMR techniques on various hydrazine-reacted PAO derivatives, we have shown that metal ion substitution occurs at the original copper binding site (25). Co^{2+} - and Zn^{2+} -substituted 9-hydrazinoacridine-PAO (acr-PAO) were prepared from acr-PAO using the same procedure.

Spectroscopic analyses. Fluorescence measurements were obtained on a 4800 series SLM-Aminco fluorometer equipped with Hamamatsu 9813QA photomultiplier tubes. All fluorescence parameters were determined at 25°C with an excitation wavelength of 340 nm. A pair of 418 nm cutoff filters were used during polarization and anisotropy measurements. Emission spectra were corrected for photomultiplier response, and then for trivial absorption due to the intrinsic absorption of acr-PAO. Fluorescence lifetimes were obtained by a phase and modulation method using glycogen as a scattering reference (26). Each phase and modulation lifetime is the average of 40 determinations performed at 30 MHz over the course of 1 week on two different sample preparations.

Ultraviolet-visible absorption spectra were obtained on a Cary 219 spectrophotometer equipped with an Apple microcomputer which was used to process the data. The Co^{2+} absorption spectrum was taken as the difference of the Co^{2+} -PAO and Zn^{2+} -PAO spectra normalized with respect to protein concentration. The molar extinction coefficients were determined from the total concentration of cobalt bound to pig PAO. A Jasco J-500 A spectropolarimeter was used for all CD measurements.

Distance calculations. Quantum yields were calculated from integrated fluorescence emission profiles using quinine sulfate as a standard (27). The quantum yield of the acridinyl moiety in pig PAO was found to be 0.0058. Peak areas determined for the quantum yields and the overlap integrals were calculated using Simpson's rule with 5-nm intervals.

According to the Forster theory of fluorescence energy transfer, the apparent distance (r') between the donor and acceptor is related to the transfer efficiency (E) by

$$r' = R_0(E^{-1} - 1)^{1/6}$$

where R_0 is the distance which results in a transfer efficiency of 50%. The transfer efficiency is experimentally determined by the following relation:

$$E = 1 - Q_{da}/Q_d = 1 - \tau_{da}/\tau_d$$

where Q and τ are quantum yields and fluorescence lifetimes of the donor in the absence (d) and presence (da) of the acceptor. R_0 is mathematically determined using

$$R_0 = (9.7 \times 10^3) [\kappa^2 n^{-4} QJ]^{1/6} (\text{\AA})$$

where Q is the quantum yield of the donor in the absence of energy transfer, n is the refractive index

of the medium, κ^2 is the orientation factor, and J , the integral ($\text{cm}^3 \text{M}^{-1}$), is calculated from the expression

$$J = \int F(\lambda) \epsilon(\lambda) \lambda^4 d\lambda / \int F(\lambda) d\lambda$$

where $F(\lambda)$ is the corrected emission intensity (arbitrary units) and $\epsilon(\lambda)$ the extinction coefficient ($\text{M}^{-1} \text{cm}^{-1}$) at wavelength λ (cm). In theory, κ^2 can assume any value between 0 and 4; in practice, the range may be more limited.

RESULTS

Inactivation of Pig PAO with Fluorescent Hydrazines

Pig PAO was titrated with various fluorescent hydrazine containing carbonyl reagents. Hydrazines that inactivated PAO included 9-hydrazinoacridine, dansyl hydrazine, DCCH, and salicyl hydrazine (Fig. 1). It was found that dansyl hydrazine and DCCH reacted with PAO more slowly than the other carbonyl reagents; all reactions reached equilibrium by 16 h. Pig PAO incubated for 16 h in the absence of carbonyl reagents showed no loss in catalytic activity. The extent of inactivation closely correlated to the amount of reagent added to the enzyme. The naphthalenemethylhydrazines were less efficient inactivators of the enzyme. In preliminary

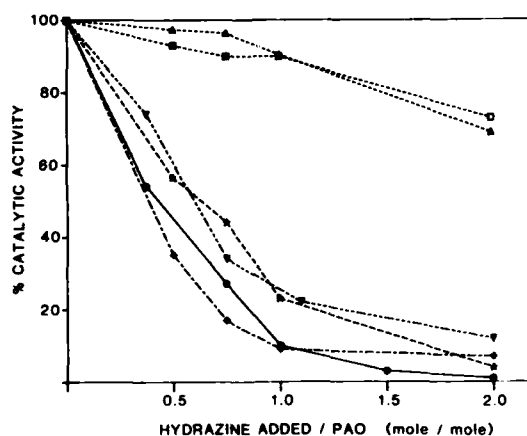


FIG. 1. Titration of pig PAO with carbonyl reagents. (Δ) 1-Naphthalenemethyl hydrazine; (\square) 2-naphthalenemethyl hydrazine; (∇) dansyl hydrazine; (\star) 7-diethylcoumarin-7-carbohydrazide; (\circ) 9-hydrazinoacridine; and (\diamond) salicyl hydrazine. Various amounts of the hydrazines were reacted with 10 μM pig PAO for 16 h, then assayed for catalytic activity.

experiments the dansyl hydrazine-inactivated enzyme was only weakly fluorescent and not studied further. 9-Hydrazinoacridine and DCCH were the only probes with suitable fluorescent characteristics for energy transfer to cobalt. 9-Hydrazinoacridine was chosen for further study because it had a longer fluorescent lifetime and has been studied more extensively. The reaction of 9-hydrazinoacridine with pig PAO could be monitored by the characteristic visible absorption peaks near 495 and 525 nm which were indicative of the product (Fig. 2).

The reaction with the enzyme as monitored by the increase in absorption at 495 and 525 nm was found to reach a maximum after 2 h coincident with 95% loss of enzymatic activity. At this point the number of remaining unreacted carbonyl groups was determined by reacting with phenylhydrazine and monitoring the increase in absorbance at 430 nm (7). The percentage of unreacted carbonyl groups determined with phenylhydrazine was in excellent agreement with the percentage of residual catalytic activity. Assuming one dimer of pig PAO possesses only one functional carbonyl cofactor and correcting for unreacted material, the extinction coefficients were determined to be 8.66×10^4 and $9.87 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for 495 and 525 nm, respectively.

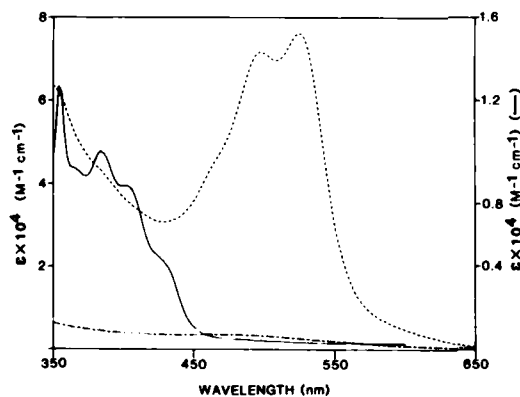


FIG. 2. Visible absorption spectra., native pig PAO, 10 μM ; —, 9-hydrazinoacridine, 25 μM ; ---, acr-PAO prepared from pig PAO, 10 μM , treated with 5 μM 9-hydrazinoacridine, then dialyzed. All solutions were in 50 mM phosphate, pH 7.2.

Spectral Characteristics

Evidence that the 9-hydrazinoacridine was covalently bound in a protected site comes from the effect of urea and pH. After denaturation and dialysis with 8 M urea, the acridinyl group remained bound to the enzyme and the visible absorption spectrum was found to be pH dependent ($pK_{a1} = 7.6$, $pK_{a2} = 10$). However, the absorption spectrum of undenatured acr-PAO was insensitive to changes in pH from 6 to 10. The structure of the enzyme as determined by uv-CD was found to be unchanged throughout this pH range. Above pH 10, changes in the uv-CD spectra revealed structural alterations in the acr-PAO concomitant with changes in the visible absorption spectrum. The fluorescence properties of acr-PAO were consistent with this trend. Acr-PAO exhibited fluorescence between 380 and 580 nm with maxima at 415 and 440 nm (Fig. 3) which did not change with pH between 6 and 10. This is in contrast to acridine which undergoes a blue shift in alkaline pH. In fact, the emission of acr-PAO resembles that for the unprotonated form of acridine found under alkaline conditions by Lakowicz (26).

Incubation of excess 9-hydrazinoacridine with pig PAO resulted in a further increase in bound fluorescent material, most likely due to binding at a second lower affinity site. Secondary binding sites can also explain the slight deviations from linearity near equal stoichiometries in the absorbance at 495 nm during titration of pig PAO with 9-hydrazinoacridine. Fluorescence polarization can be used to determine the amount of 9-hydrazinoacridine bound to the enzyme and can sometimes distinguish between different modes of binding. By comparing fluorescence polarization values of the different samples, it was found that dialysis against 50 mM sodium phosphate buffer, pH 7.2, was sufficient to remove excess label. The addition of up to 8 M urea or 0.5% octylglucopyranoside did not cause additional label to be removed. It was also observed that secondary binding could be reduced by limiting the amount of the hydrazine used

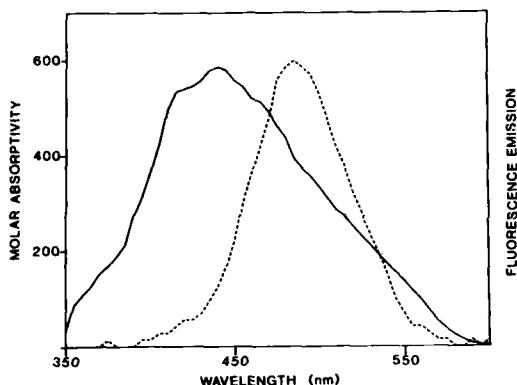


FIG. 3. Spectral overlap between ---, the absorption spectrum of Co-substituted pig PAO calculated as described under Methods and —, the fluorescence emission of acr-PAO excited at 340 nm.

in the reaction. We chose to incubate pig PAO with 0.5 mol 9-hydrazinoacridine per mole dimer, which inactivated about 50% of the enzyme. Under these conditions all of the 9-hydrazinoacridine remained bound to the enzyme even after denaturation with 8 M urea.

Metal Ion Depletion and Fluorescence Energy Transfer Calculation

Since the copper present in the enzyme has visible absorption, we removed the copper using cyanide under anaerobic conditions (23). When an energy acceptor was desired, cobalt was added to the metal depleted acr-PAO. Alternately for the system lacking an energy acceptor, zinc was added to the metal-depleted acr-PAO. The lifetimes in Table I represent the average of the phase and modulation values, which were indistinguishable. Lifetime measurements made at 18 and 6 MHz were consistent with the results obtained at 30 MHz, further confirming that the fluorescence lifetime was homogeneous. The lifetimes of the cobalt-substituted and zinc-substituted acr-PAO were 2.7 and 3.4 ns, respectively. A transfer efficiency of 22% was obtained from these values. Integrating their emission spectra results in relative quantum yields of 0.74 and 1.00 for the Co^{2+} and Zn^{2+} forms, respectively, corresponding to a transfer ef-

iciency of 26% which is consistent with the value obtained by lifetimes.

The difference absorption spectrum for cobalt in pig PAO overlaps the acr-PAO emission spectrum over the range of 380 to 570 nm (Fig. 3). Assuming a value of 2/3 for κ^2 , R_0 is 10.4 Å and the apparent distance between the donor and acceptor is 13.0 Å. Since Co^{2+} was used as an energy acceptor, κ^2 may range from 1/3 to 4/3. R_0 can range from 9.3 to 11.7 Å, and the actual distance from 11.7 to 14.7 Å. These distances are in excellent agreement with recent ^{19}F NMR experiments by Williams and Falk (25) where the center of the aromatic ring of phenylhydrazine was estimated to be 12.6 Å from the copper ion.

DISCUSSION

Hydrazinoacridine has been shown to inactivate PAO by covalently reacting with the carbonyl function near the active site. It has been demonstrated that 1 mol of phenylhydrazine as well as several trifluoromethyl derivatives completely inactivates pig PAO by binding covalently to the amine substrate binding site (5, 6, 7, 25). Dansyl, acridinyl, salicyl, and coumarin hydrazines also inactivate PAO. In several cases, slightly more than equimolar amounts of those reagents are required for complete inactivation. This is probably due to a slower reactivity with the enzyme

TABLE I
SUMMARY OF ENERGY TRANSFER PARAMETERS

Lifetimes	
Zn-acr-PAO ^a	3.4 ns
Co-acr-PAO ^a	2.7 ns
Transfer efficiency	22%
Overlap integral	$1.164 \times 10^{-15} \text{ cm}^3 \text{ M}^{-1}$
R_0 (2/3)	10.4 Å
Most probable distance, r' (2/3)	13.0 Å
Minimum distance, r' (1/3)	11.7 Å
Maximum distance, r' (4/3)	14.7 Å

^a Zn-acr-PAO and Co-acr-PAO, zinc- and-cobalt-substituted forms of acr-PAO, respectively.

and the greater probability of the probe decomposing or reacting with components of the buffer. In spite of this, the majority of the label reacts with PAO, behaves similarly to phenylhydrazine, and therefore acts as an analog of the substrate, benzylamine. The covalent insertion of a fluorescent reporter group in the active site of PAO allows one to study various aspects of the structure of PAO. The lack of pH dependence of the spectrum of the native form as contrasted with the pH dependence of the denatured form of acr-PAO demonstrates that the probe resides in a protected site. In addition to determining characteristics of the binding site, fluorescence measurements allow the estimation of the distance from the reporter group to the metal.

Dipole-dipole energy transfer such as we have described here is dependent on the isotropy and the orientation of both the donor and acceptor moieties. When one of the energy transfer moieties is isotropic, as is the energy acceptor, cobalt, the limits of κ^2 are 1/3 and 4/3 (28, 29). If the other energy transfer moiety is either isotropic or anisotropic but can rotate rapidly and freely in relation to its lifetime, then $\kappa^2 = 2/3$ (30). Distances were calculated for all the above values of κ^2 (Table I).

Although PAO contains two copper ions, only one is essential for full activity in the case of the bovine enzyme (22, 24). Furthermore, the two coppers are nonequivalent and separated by at least 11 Å from each other as determined by EPR (15). One of the experimentally determined values required for the calculation of the overlap integral is the extinction coefficient of the acceptor. The absorption of cobalt was determined as the difference between cobalt-substituted PAO and zinc-substituted PAO. Extinction coefficients were calculated from the absorption spectrum using the total concentration of cobalt bound to pig PAO which was 1.8 mol per mole dimer. Since we were unable to determine the occupancy of cobalt in each of the two sites, we assumed an average distribution and that each site contributed equally to the absorption. Although this

may not be strictly valid in view of the EPR-determined asymmetry (16, 20, 21), this assumption introduces only a small error in the overlap integral calculation. If only one of the cobalt ions is involved in the absorption, then the extinction coefficients shown in Fig. 3 would be doubled. Since the distance is related to the sixth root of the overlap integral, then the distance would be in error by a factor of $2^{1/6}$ or 1.12.

Even though in the present study certain assumptions were employed in the calculation of the distances, a different set of assumptions used previously to determine the distance by NMR gave similar results. One of the assumptions previously addressed in this study is that the cobalt ions added back to the metal-depleted enzyme occupy the native enzyme-copper binding sites. Another assumption is that metal substitution does not alter the fluorescence emission spectrum of the modified enzyme. The fact that the distance from the copper to the hydrazine binding site was found to be the same distance as that determined from the cobalt to the hydrazine binding site in this study sug-

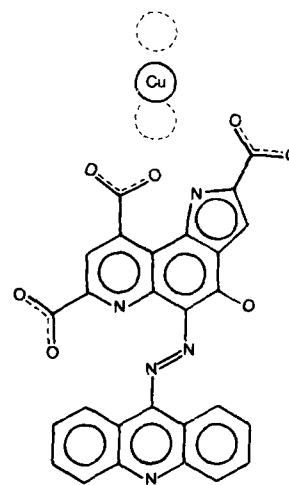


FIG. 4. Proposed chemical structure of the active site of pig PAO. Since the state of ionization and the mode of attachment of the cofactors are not known for PAO, the drawing omits protons. The dotted circles represent the position of the copper at 11.7 and 14.7 Å from the acridine ring; the solid circle is the position of the copper at 13 Å.

gests that these assumptions are warranted.

The structure depicted in Fig. 4 is a composite of information from several sources. The identity and structure of the cofactor was determined by several sources (10-14, 31). The nature of the bonds between hydrazine reagents and the cofactor was drawn from X-ray crystallographic and resonance Raman spectroscopic evidence (31, 32). The relative orientation of the copper and carbonyl reagent was defined in the present work and by NMR (25). An alternative structure (not shown) would result from a rotation of 180° about the C-N bond of the azo group. In this conformation, the center of the inhibitor ring system and concomitantly the position of the Cu atom would be shifted about 3.3 Å to the right. This would place the copper atom in close proximity to the carbonyl functional group at the 2-position of the PQQ cofactor. Based on these measurements, the copper resides at least 3.5 Å away from the PQQ ring system. This rules out any involvement of the ring nitrogen of PQQ in the coordination of the copper as suggested by others (33, 34).

It has been reported that Cu²⁺ is involved in the oxidation of a reduced form of the enzyme, although it remains in the cupric form during catalysis. It is unlikely that copper would directly interact with the substrate since their separation is too great. It is more likely that the oxidation occurs through some region of the cofactor distant from the substrate binding site, otherwise a large conformational change or additional electron carrier would be required. The distance between the copper and the PQQ ring depicted in Fig. 4 precludes any direct interaction of the two cofactors. However, copper could be involved in the binding or positioning of oxygen for an interaction between oxygen and PQQ.

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